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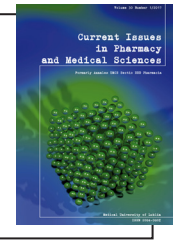

Tlr2, *Tjp1*genes expression during wound healing dynamics – with melanin treatment

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ABSTRACT

Wound healing is the complex and dynamic process of replacing devitalized and missing cellular structures and tissue layers. We have previously shown that melanin, herein, produced by the Antarctic black yeast fungi *Pseudonadsoniella brunea* (*Nadsoniella nigra sp. X-1*), has expressed a cyto-protective effect, promoted rapid wound healing of various ethiology and can be offered as a new dermatropic drug. The current study was conducted on a rat model of purulent necrotic wound. In each model, one group was a control, while in the others, wound healing occurred without drug application or with administration of 0,5% carbopol or with both 0,5% carbopol and 0,1% melanin. The pro-oxidant-antioxidant balance in skin gomogenate in dynamics on 3, 6, 9, 14 and day of full epithelization was estimated using the spectrophotometric biochemical method. Moreover, so as to understand the role played by the *Tlr2* and *Tjp1* in the process of wound healing and scar formation, *Tlr2*, Tjp1gene expression and genetic mRNA was determined with quantitative RT-PCR. The application of our pharmacological composition stimulated the decrease of *Tlr2* and *Tjp1* gene expression against the background of suppression of free radical processes (reduction of superoxide anion radical content) with epithelization and without scarring. The results of this study have shown the positive effects of melanin on wound healing. The obtained results indicate the advisability of applying melanin for the treatment of inflammatory processes.

INTRODUCTION

The human epidermis is a multilayered structure consisting of four series of differentiated layers. Beyond other functions, these layers serve as barriers to outside infective agents. Temporal distortion of the skin properties are caused by scratches, cuts, burns, organic solvents, detergents, endogenous or exogenous proteases of microorganisms [13].

Such distortion opens up the body to infection.

The process of free radical oxidation is a component of the metabolic activity of cells constantly occurring in living organisms. The main mechanism of free radical process activation is to increase the formation of reactive oxygen species (ROS). Their damaging effect is brought about mainly by the further stimulation of free radical oxidation that leads to oxidative stress, which is manifested by the accumulation of toxic products that damage the molecules, cell membranes and tissues, as well as disrupt wound reparative processes [7,17]. Toll-like receptors are a class of cellular receptors with a transmembrane domain which play a key role in innate immunity. They recognize the conserved structures of microorganisms activated against the cellular branch of the immune system, and, as was recently shown, regulate the barrier function of the skin [14,8]. The *Tlr2* gene (encoding the Toll-like receptor 2) is expressed in enterocytes, colonocytes, nonspecific immunity cells (macrophages, mastocyte, etc.), keratinocytes, sebaceous glands and so on. TLR2, the product of this gene, plays a key role in identifying the components of the cell wall of gram-positive bacteria

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(e.g., *Staphilococcus aureus*) and maintaining tolerance to own microflora [10].

The cytoplasmic protein plate – ZO-1 (*Zonula occludens*-1 gene encoded Tjp1), together with its partner – occludin, forms one of the corresponding protein complexes protein-associated solid contacts (ZO) [14]. If these tight junctions proteins are destroyed by the influence of pathogens or physical damage or due to calcium deficiency, there is a violation of the barrier function of the skin, which, in turn, can lead to a number of skin lesions [5,9,12]

We have previously shown that yeast melanin (*Nadsoniella nigra*) has expressed a cyto-protective effect, promoted rapid wounds healing of various etiology and can be offered as a new derma tropic-drug [8]. A pharmacological composition (0.1% melanin, dissolved in 0.5% Carbopol) has been successfully probed on animal models [16,6]. In the current study, the melanin was produced by the Antarctic black yeast fungi *Pseudonadsoniella brunea* (*Nadsoniella nigra sp. X-1*), the source of which was samples obtained originally off the vertical cliffs of the island of Galindez (Ukrainian Antarctic Station "Akademik Vernadsky").

Our purpose was to analyze the intensity of free radical processes and Tlr2, Tjp1 gene expression during wound healing, particularly after administration of melanin-based pharmacological compositions.

MATHERIALS AND METHODS

The study was approved by the Bioethical Commission of ESC "Institute of Biology" of Taras Shevchenko National University of Kyiv on 26 June 2013. In our study, all animals were handled humanly according to the rules outlined in "Guide for the Care and Use of Laboratory Animals" (2011), and in the Order of Ukraine № 3447-IV "About defense of animals from abusive handling" from 21 February 2006.

All experiments were carried out on white non-strain female rats (weigh 200-250 g, n = 96). These were placed within four groups. In each model, animals without experimental skin wounds were used as a control (first group). When the animals were injured, they were anesthetized by sodium thiopental ("BiochemieGmbH", Austria), at a dosage of 50 mg / kg. In the second group, healing occurred without drug application, whereas the wounds of the rats of third group were treated only with 0,5 % carbopol, while animals of the fourth group received 0,1% melanin (produced by the Antarctic black yeast-like fungi *Pseudonadsoniella brunea* (*Nadsoniella nigra sp. X-1*)) dissolved in 0,5% carbopol for wound treatment.

Chemical skin burns were induced by the introduction of 0,1 ml $CaCl_2$. Attention was payed to the standardization of wounds received, the size of which did not exceed 400 mm2. After 4-5 days, necrotomy of the affected area was performed and then treatment of wounds was begun until healing [2].Our previous studies found that rapid wound healing of various etiologies using melaninin occurred in the initial phase of the regeneration of the skin. Herein, analysis of active contraction of the wound surface in dynamics was evident on days 3, 5, 7, 9, 14, 21 and on the 30th day. Beyond the aforementioned, melanin revealed a strong bactericidal effect on *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*, hence, demonstrating the appropriate application of the drug for the treatment of infectious inflammatory processes [16].

Determination of the intensity of superoxide anion radical production

The intensity of generation of superoxide anion radical in homogenates was determined by the material accumulation of HTT-formazan [15,1]. The principle of the method is the ability to interact with superoxide anions 2,3-bis(2-methoxy-4-nitro-5-sulfofenil)-2N-tetrazol-5-2-*carboxyanilid* (HTT) to form a soluble complex, HTT-colored formazan, having a peak absorbance at 470 nm.

Quantitative RT-PCR

RNA was isolated following Chomczynski and Sacchi [3]. Conditions of cDNA synthesis and quantitative Real-time PCR (qRT-PCR) were according to the provided instruction of the SYBR Green I kit "Thermo Scientific Verso SYBR Green 1-Step qRT-PCR ROX Mix" ("Thermo Scientific", Lithuania): cDNA synthesis 50°C – 30 min; initial denaturation 95°C – 15 min; following 40 cycles: denaturation ДНК 95°C – 15 s; annealing 50°C – 35 s; extension 72°C – 30 s; final extension 72°C – 5 min. Primers were designed into exon-intron junctions to avoid amplification of genomic DNA. The primers used were (designed using Primer-BLAST): for *Tlr2* – forward – TGGTAGTTGTGGGTTGAAGC and reverse – GACAGAGAAGCCTGATTGGAG; for *Tjp1*: forward – CCATCTTTGGACCGATTGCTG, reverse – TAATGCCCGAGCTCCGATG; for *Actb* (as an endogenous control gene) – forward – TGGGACGATATGGAGAAGAT and reverse – ATTGCCGATAGTGATGACCT. We ran at least 3 replicates for each gene, RNA sample and every primer. The melting curve analysis was carried out to assess whether the intercalating dye in qRT-PCR assays produced single, specific products without formation of primer dimers.

The relative quantification was calculated by the comparative C_T method («$\Delta\Delta C_T$ Method» : the data were analyzed using the equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ = [C_T of target gene – C_T of housekeeping gene]treated group − [C_T of target gene – C_T of housekeeping gene] untreated control group. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression, normalized to the housekeeping gene (*Actb*), and relative to the untreated control. In a validation of $\Delta\Delta$CT calculation, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal (Ex = ($10^{-1/slope}$) – 1 × 100 %), the absolute value of the slope of ΔC_T vs. log input is < 0,1.

Statistics

Statistical processing of experimental results was carried out in "GraphPad Prism 5" ("GraphPad Software Inc.", USA). The type of in-group data distribution was verified via the Shapiro-Wilk test. As data were distributed normally ($p > 0,05$), two-way ANOVA was conducted to determine the significance of difference between means, with Bonferroni post test. Difference between means was judged as statistically significant if $p \leq 0,05$. Mean and standard deviation (SD) were calculated for each group.

RESULTS AND DISCUSSION

Superoxide anion in a group of animals with purulent-necrotic wounds was higher at 2.2 ($p \leq 0.001$), 2.8, 2.5 ($p \leq 0.0001$) and 1.9 times ($p \leq 0.05$) on the 3, 6, 9 and 14 days of healing, respectively, compared to control (Table. 1).

Table 1. The accumulation of superoxide radicals in the dynamics of wound healing and during pharmacological composition administration(M ± SD, n = 7)

Group of animals	Time, day	HTT-formazan nmol × mg protein^{-1}
Control	-	28.9±2.31
Wound	Purulent necrotic wound	
	3	63.6±5.91***
	6	80.9±7.15****
	9	72.3±6.52****
	14	54.9±5.94*
	Complete epithelization	34.7±2.90
Wound + Carbopol	3	61.6±5.98***
	6	82.9±8.15****
	9	75.3±7.52****
	14	53.9±5.74*
	Complete epithelization	29.7±2.90
Wound +melanin	3	61.9±6.23***
	6	52.5±6.71$^{*/\#\#}$
	9	42.5±4.74$^{\#\#}$
	14	31.0±4.31$^{\#}$
	Complete epithelization	30.79±3.51

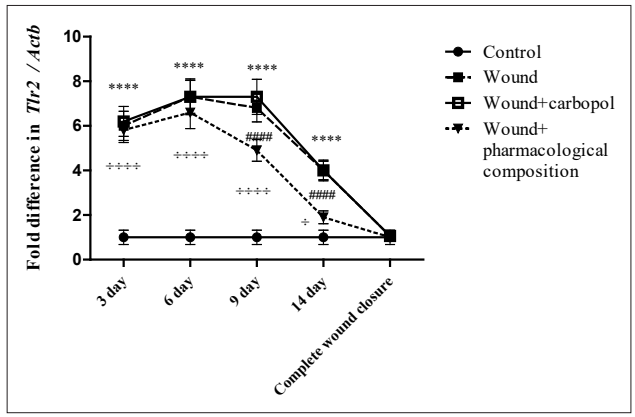
Notes: **** - p ≤ 0.0001, *** - p ≤ 0.001, ** - p ≤ 0.01, * - p ≤ 0.05 relative to control; ### - P ≤ 0.001, ## - p ≤ 0.01, # - p ≤ 0.05 wounds inflicted with pharmacological composition relative to animals with wounds

The levels of gene expression in the second and third group of rats also were not significantly different. At the same time, the animals whose wounds were treated with pharmacological composition, this indicator was at 1.6 ($p \leq 0,01$), 1,7 ($p \leq 0.01$) and 1.8 times ($p \leq 0.05$) lower on the increased relative to controls: at 2.1 ($p \leq 0.001$) and 1.8 ($p \leq 0.05$) 3 times on the 6 day of healing, respectively, and on the 9 day returned to control values.

The level of mRNA gene was at the control in the second, third and fourth groups of animals in the complete wound epithelialization. The complete wound closure in rat group with purulent necrotic wounds without treatment was on the 38.1 ± 0.5 days, while rats whose wounds were treated with melanin – on the 36.0 ± 0.7 day [16].

Gene expression level of *Tlr2* group of animals with purulent necrotic wounds was 6, 7.3, 6.8 and 4 times higher ($p \leq 0.0001$) on the 3, 6, 9 and 14 days of healing in comparison with the control (Figure 1).

The levels of expression of this gene in the second and third group of rats was not significantly different. However, in animals whose wounds were inflicted with the pharmacological composition, the figure was 1.4 and 2.1 times ($p \leq 0.0001$) lower on the 9th and 14th day, respectively, than in the second group of animals, and was less increased if compared to control: 1.9 times ($p \leq 0.05$) on the 14th day of healing. The level of mRNA gene was at the control values

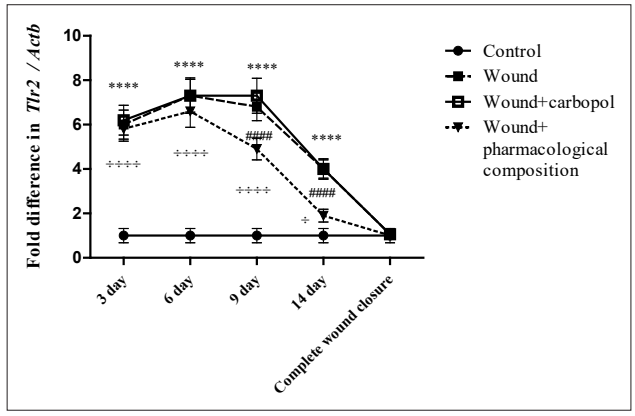


**** - P ≤ 0.0001 wound relative to control; #### - P ≤ 0.0001 wound inflicted with pharmacological composition relative to animals with wounds; ÷÷÷÷ - p ≤ 0.0001, ÷ p ≤ 0.05 wounds inflicted with pharmacological composition compared to control

Figure 1. The level of mRNA Tlr2 gene expression in dynamics of purulent necrotic wounds healing and with the introduction of melanin

in the second, third and fourth groups of animals at the time of the full wound closure.

In the animals with purulent necrotic wounds, the level of *Tjp1* gene expression was 5.2, 3.8, 3.2 and 2.4 times ($p \leq 0.0001$) lower on the 3rd, 6th, 9th and 14th days of healing, respectively, compared with control (Figure 2).



**** - P ≤ 0.0001 wound relative to control; #### - P ≤ 0.0001, ### - p ≤ 0.001 wounds inflicted with pharmacological composition relative to animals with wounds; ÷÷÷÷ - p ≤ 0.0001 wound inflictedwith pharmacological composition compared to control

Figure 2. The level of mRNA Tjp1gene expression in dynamics of purulent necrotic wounds healing and with the when melanin administered

The levels of mentioned gene expression in the second and third groups of rats did not differ significantly. In animals whose wounds were inflicted with the pharmacological composition, the figure was 1.6 ($p \leq 0.001$) and 2.2 times ($p \leq 0.0001$) higher on the 9th and 14th days, respectively, than in the second group of animals, and was less reduced relative to control, at 5.1, 3 and 2 times ($p \leq 0.0001$) on the 3rd, 6th and 9th days of wound healing, respectively, and on the 14th day, returned to control values. In addition, mRNA Tjp1 level was at control values in the second, third and fourth groups of animals at the time of complete closure of the wound.

Healing is a dynamic process, which on the one hand, displays excessive inflammation, on the other, remediates temporal damage or chronic wounds [13,10,16,6]. The

superoxide anion radical is directly involved in the initiation of free radical processes. Its generation is the starting element of cascade reactions that lead to the emergence of other ROS. The growth of superoxide anion radical content under such conditions as healing of purulent necrotic wound surfaces after burns caused by calcium salt solution is due to the generation of mitochondrial and microsomal electron transport chain epithelial cells.

Epidermal close contacts consist of transmembrane proteins. These are dynamic structures that reduce or increase density in response to both endogenous signals from the epithelial and subepithelial compartments, and the effect of exogenous agents: bacteria and allergens. Changes in the expression of any gene encoding protein of tight junctions has a significant impact on the integrity of tight junctions [10,5]. In our study, we found a decrease in mRNA Tjp1genes encoding the cytoplasmic protein ZO-1 plate in cut and purulent necrotic wounds in rats. Similar results were obtained in patients with atopic dermatitis, in animal models of plane cut wounds and in the culture of human neonatal primary keratinocytes [10,5]. Restoration of tight junctions in keratinocytes was induced by the impact of innate immunity receptors TLR2.

Increased expression of *Tlr2* in the neighboring cells of the epidermis (in the cellularlayer lowertight junctions) during the destruction of the skin barrier is necessary to generate a quick response to the pathogens that proliferate in the place of the wound defect [10,12]. Such response facilitates the early formation of the tight junctions in the keratinocytes which form the basis of a monolayer of dermal wounds. Early formation of tight junctions prevents further leakage of serum, and, thus, limits the amount of nutrients and adhesion molecules that promote excessive growth of invasive microorganisms. It also minimizes the loss of water, preventing wounds dehydration [10]. Further research is, however, needed to identify the mechanisms by which TLR2 helps to restore the barrier function of tight junctions. It is suggested that interleukin 1 and TNF-α may be important mediators in the TLR2-mediated repair of the skin barrier. Still, some studies have shown that some miRNA inhibit the synthesis of TLR2 [10,9].

In this study, while under conditions of melanin-based treatment of wounds, we saw less reduction in *Tjp1* gene expression and the lowering of the *Tlr2* value against a background of the suppression of superoxide radical accumulation in the dynamics of healing. We also saw less downregulation of *Tlr2* and more rapid return of *Tjp1* gene expression to control levels.

The reduction of *Tlr2* expression can be explained by melanin having a strong bactericidal effect on abnormal flora: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* [16]. This indicates that TLR2 has reparative activity for accelerating the recovery of the epidermal barrier and for limiting the growth of pathogens in the healing of wounds of various etiologies under the conditions of melanin pharmacological treatment. In previous studies, we have shown an increase in gene expression in the healing of cut wounds and purulent necrotic wounds. When using melanin under the same conditions, the expression level of these genes against the background of scarring, was decreased [6]. As for the possible mechanisms of melanin influence as a polyphenolic compound on the analyzed gene expression, during the healing of skin lesions of various etiologies, first of all, its frank cytoprotective effect should be noted. Melanin reduces the activity of lipid peroxidation, increases the activity of enzymes of antioxidant system and prevents DNA damage. What is more, it influences the cytokines production: TNF-α, IL-6, VEGF, etc. by, for example, its impact on the expression of nuclear receptors PPAR [4]. Furthermore, melanin increases eNOS expression and the outflow of anti-inflammatory cytokines so as to reduce the intensity of inflammation in wound healing [6,11,15,16].

Thus, our results indicate the advisability of applying pharmaceutical compositions based on melanin for the treatment of inflammatory processes in the skin wounds.

CONCLUSIONS

We have shown the decrease of *Tjp1*gene expression level in the healing of purulent necrotic wounds against a background of free radical process activation (the growth of superoxide anion radical content). Restoring the expression level of this gene was mediated by the increase of *Tlr2* gene expression level in purulent necrotic wounds. After applying the melanin under the same conditions, the expression level of *Tjp1* quickly approached the reference value in the specified wound models. The results of this study have shown the positive effects of melanin on wound healing. The obtained results indicate the advisability of applying melanin for the treatment of inflammatory processes.

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